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Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex**

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Supporting information:

Three supplementary tables and a supplementary figure are available with the online version of this paper.

Abbreviations:

Bcc – *Burkholderia cepacia* complex; CF – cystic fibrosis; CGD – chronic granulomatous disease; EPS – exopolysaccharide; HPAE-PAD – high-performance anion-exchange chromatography with pulsed amperometric detection; TFA – trifluoroacetic acid

Abstract

The species which presently comprise the *Burkholderia cepacia* complex (Bcc) have multiple roles which include soil and water saprophytes, bioremediators, and plant, animal and human pathogens. Since the first description of pathogenicity in the Bcc was based on sour skin rot of onion bulbs, this study focused on growth on onion tissue to investigate the “natural phenotype” of these organisms. Many Bcc isolates, which were previously considered to be nonmucoid, produced copious amounts of exopolysaccharide (EPS) when onion tissue was provided as the sole nutrient. EPS production was not species-specific, was observed in isolates from both clinical and environmental sources and did not correlate with the ability to cause maceration of onion tissue. Chemical analysis suggested that the onion components responsible for EPS induction were primarily the carbohydrates sucrose, fructose and fructans. Additional sugars were investigated, and all alcohol sugars tested were able to induce EPS production, in particular mannitol and glucitol. Representatives of the *B. cenocepacia* ET12 lineage did not produce EPS under any growth conditions investigated. This finding correlated with the presence of an 11 bp deletion in the *bceB* gene which encodes a glycosyltransferase responsible for the catalysis of the first step of the assembly of the EPS repeat unit. These novel and surprising insights into EPS biosynthesis highlight the metabolic potential of the Bcc and show that a potential virulence factor may not be detected by routine laboratory culture. Our results also highlight a potential hazard in the use of inhaled mannitol as an osmolyte to improve mucociliary clearance in individuals with cystic fibrosis.

Introduction

The genus *Burkholderia* includes three closely-related microbial species that highlight diverse evolutionary adaptation to different niches and hosts. *Burkholderia mallei* is a solipid-specific pathogen which only occasionally infects humans. *B. pseudomallei* is a free-living soil microbe and the causative agent of the sub-tropical human disease melioidosis. The species which presently comprise the *Burkholderia cepacia* complex (Bcc) have multiple roles which include soil and water saprophytes, rhizosphere parasites, bioremediators, plant growth promoters and plant, animal and human pathogens. Bcc are particularly associated with life-threatening respiratory infections in patients with chronic granulomatous disease (CGD), and are the most potentially virulent, transmissible and inherently resistant microbes to have emerged as cystic fibrosis (CF) pathogens in recent decades (Govan, 2006; Mahenthiralingam *et al.*, 2005).

Although most species within the Bcc produce a variety of putative virulence factors, the role of these factors in the pathogenesis of human infection is unclear (Mahenthiralingam *et al.*, 2005). Evidence from various model systems (mouse, rat, plant and nematode) suggests that the importance of individual virulence factors, or combinations of factors, depends on the infection model used (Bernier *et al.*, 2003). In addition, studies of Bcc infections in CF patients also suggest a key role of host/pathogen interactions since clinical outcome in

individual patients cannot be predicted even during epidemic outbreaks when multiple patients are infected by the same strain (Govan *et al.*, 1993). The first description of pathogenicity in the Bcc was based on sour skin rot of onion bulbs (Burkholder, 1950). In this study, we returned to this plant host to investigate the onion-associated phenotype of the Bcc, and reveal a link between growth conditions and exopolysaccharide (EPS) production.

Exopolysaccharide is a putative Bcc virulence factor which is involved in persistence of the bacteria in CF lungs (Conway *et al.*, 2004), interactions with antimicrobial peptides (Herasimenka *et al.*, 2005) and the formation of biofilms (Cunha *et al.*, 2004). The EPSs of *Burkholderia* species have recently been comprehensively reviewed (Goldberg, 2007). Recent studies (Zlosnik *et al.*, 2008) have also challenged the previous belief that mucoid, EPS-producing colonial morphotypes of Bcc are rare in both environmental and clinical isolates (Govan & Deretic, 1996). Other studies have shown that mucoid Bcc isolates mostly synthesise one type of EPS, with a highly branched heptasaccharide repeating unit, which was named cepacian (Moreira *et al.*, 2003; Sist *et al.*, 2003). EPS production has been shown to increase when the Bcc are grown in mannitol-rich yeast extract medium (MYEM) (Sage *et al.*, 1990; Zlosnik *et al.*, 2008).

Here we report the novel observation that many Bcc isolates, found to be nonmucoid on typical culture media, produce copious amounts of EPS when onion tissue is provided as the sole nutrient. Chemical and molecular analyses suggest that EPS biosynthesis is strain-specific and that the plant compounds responsible are primarily sugars and sugar alcohols. We show that the EPS phenotype on onion media is associated with the previously described *bce* cluster (Moreira *et al.*, 2003), thought to be involved in cepacian biosynthesis.

Methods

Bacterial strains and culture conditions. Bcc isolates used in this study are described in Table 1 and include 16 isolates from the two published Bcc strains panels (Coenye *et al.*, 2003; Mahenthiralingam *et al.*, 2000). Additional Bcc strains investigated included *Burkholderia pyrrocinia* BTS7, *Burkholderia cenocepacia* BTS2, as well as 19 *Burkholderia multivorans*, 14 *Burkholderia cenocepacia* IIIA and 11 *Burkholderia cenocepacia* IIIB isolates from our collection. Isolates were recovered from storage at -80 °C by subculture on nutrient agar (NA; Columbia base agar, Oxoid) and subsequently grown on media composed of 1.5% (w/v) bacteriological agar (Oxoid) containing 2% (w/v) sugars and fractions from various isolation methods below.

Sugars and other related chemicals were purchased from: Sigma-Aldrich, BDH AnalaR, MP Biochemicals, Fluka BioChemika, or Acros Organics.

Table 1. Exopolysaccharide biosynthesis of *Burkholderia cepacia* complex species when grown on agar supplemented with various substrates.

Species	Strain	Source	YE	Onion	Glucose	Sucrose	Fructose	Inulin	Glycerol	Mannitol	Glucitol	Ribitol	Inositol	Mannose
<i>B. cepacia</i>	ATCC25416	Onion	-	++	-	++	+++	-	+++	+++	+++	+++	+++	+
<i>B. cepacia</i>	CEP509	CF	-	-	-	+	++	+	++	++	+	+	+	-
<i>B. multivorans</i>	C1576	CF	-	++	-	+	+++	+	++	+++	+++	+++	+++	-
<i>B. multivorans</i>	ATCC 17616	Soil	-	++	-	+	+++	+	++	+++	+++	+++	+++	-
<i>B. cenocepacia</i>	J2315*	CF	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. cenocepacia</i>	K56-2*	CF	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. cenocepacia</i>	BC7*	CF	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. cenocepacia</i>	PC184	CF	-	++	-	+	++	+	+	++	+	+	+	-
<i>B. cenocepacia</i>	BTS2	CF	-	++	-	+	++	+	+	++	++	+	++	-
<i>B. stabilis</i>	LMG14294	CF	-	-	-	-	+	-	-	+	-	-	-	-
<i>B. vietnamiensis</i>	LMG10929	Rice	-	+++	-	++	+++	+	+++	+++	+	-	+++	++
<i>B. vietnamiensis</i>	PC259	CF	-	++	-	+	+++	+	++	+++	+	-	-	-
<i>B. dolosa</i>	E12	CF	-	++	-	-	+++	-	++	++	-	+	+++	-
<i>B. ambifaria</i>	AMMD	Soil	-	+++	-	+	+++	+	+++	+++	+++	+++	+++	-
<i>B. anthina</i>	W92 ^T	Soil	-	+++	-	-	+++	+	+++	+++	+++	+++	+++	++
<i>B. anthina</i>	C1765	CF	-	+	-	-	+++	-	+++	+++	-	+++	+	+
<i>B. pyrrocinia</i>	BTS7	CF	-	+++	-	+	+++	+++	+++	+++	+++	+++	+++	+
<i>B. pyrrocinia</i>	C1469	CF	-	++	-	++	++	+	++	+++	+++	+++	++	-

All strains tested are from the two published Bcc panels (Coenye *et al.*, 2003; Mahenthiralingam *et al.*, 2000) except BTS2 and BTS7 that were donated by Paola Cescutti. EPS production was scored on a scale from – (no EPS) to +++ (very mucoid). Mucoid growth described as +++ is shown in Figure 1b. *ET12 isolates. YE, yeast extract agar.

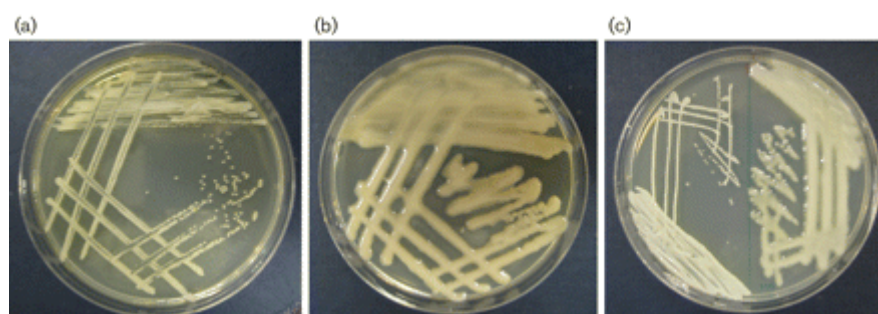


Figure 1. Growth of *B. ambifaria* AMMD on (a) nutrient agar (non-mucoid); (b) onion agar (mucoid), and (c) comparison of AMMD *bceB* mutant (left) and AMMD wild-type (right) on mannitol agar.

Onion maceration. White onion slices were placed in Petri dishes and inoculated with stationary phase Bcc cultures (10^6 cfu) that had been cultured overnight in 2.5% (w/v) nutrient broth No 2 (Oxoid) with 0.5% (w/v) yeast extract. The onions were left at 30 °C for 5 days. The results were assessed by eye, and onion maceration recorded as positive or negative.

Onion extract agar. Peeled white onions (1 kg) were chopped, homogenised in a blender at room temperature, and filtered through muslin. The filtrate was filter-sterilized through a 0.22 µm filter and lyophilised to give a yellow sticky powder (typical yield 62 g). Twenty grams of lyophilised onion extract and 15 g bacteriological agar were made up to 1 litre distilled H₂O, then autoclaved at 121 °C for 15 min. Strains were subcultured onto onion agar and incubated for 72 hours at 30 °C. Mucoidy was recorded on a scale ranging from nonmucoid (-) to very mucoid (+++).

Sugar agar. Sugar agar contained 20 g of the sugar of interest, 2 g yeast extract, and 15 g bacteriological agar dissolved in 1 litre distilled H₂O (Sage *et al.*, 1990). The sugars used were as follows: D-fructose, D-galactose, D-mannitol, D-glucose, glycerol, lactose, L-rhamnose, D-mannose, maltose, sucrose, *myo*-inositol, ribitol (adonitol), and D-glucitol (sorbitol). The fructan polysaccharide inulin was also tested.

As a control isolates were grown on bacteriological agar containing 0.2% (w/v) yeast extract alone.

Reverse-phase chromatography. Twenty millilitres of onion extract, 2% (w/v) in distilled H₂O, was loaded onto a pre-packed C8 column (10 g/60.0 ml, Varian, Anachem) and bound material was eluted stepwise using 3 concentrations of methanol (20% (v/v), 50% (v/v) and 80% (v/v); Fisher Scientific). Each fraction was lyophilised, dissolved in distilled H₂O and incorporated into 1.5% (w/v) bacteriological agar.

Ethyl acetate partitioning. To separate any lipids, non-polar, non-acidic and polar compounds, the resolubilised onion extract was brought to pH 7.0 using NaOH and partitioned against ethyl acetate (Fisher Scientific) at 1:1 (v/v). The phases were separated, and the aqueous phase was brought to pH 2.0 using HCl and the extraction was repeated. Following both extractions, the organic and aqueous layers were evaporated or lyophilised respectively, and then redissolved in distilled H₂O, pH adjusted to 7.0, and incorporated into 1.5% (w/v) bacteriological agar.

Acid hydrolysis. The aqueous phase residue of the ethyl acetate partitioned onion extract was redissolved in distilled H₂O (50 mg ml⁻¹) and was hydrolysed in 2 M TFA (trifluoroacetic acid; Sigma) at 60 °C or 120 °C for 1 h.

Paper electrophoresis. To fractionate the extract based on the presence or absence of functional groups, the freeze-dried aqueous phase of the onion extract was weighed and resuspended in distilled H₂O to a final concentration of 50 mg ml⁻¹. One millilitre was loaded at a centre origin of a Whatman No.1 paper (57×42 cm). The following standards were added in the margins of the paper: glucose 6-phosphate, glucose,

glucosamine, methyl green (Sigma) and orange G (BDH). Electrophoresis was conducted at 1 kV for 20 min, in volatile buffers at pH 2.0 or 6.5 with white spirit or toluene as coolant (Fry, 2000).

Paper chromatography. One millilitre aliquots of each of the redissolved aqueous phase of the onion extract and the TFA hydrolysed samples (50 mg ml^{-1}) were chromatographed on Whatman No1 paper alongside markers [ferulic acid, rhamnose, glucose, lactose, mannose, galactose, fructose, mannitol, and glycerol] in butan-1-ol:acetic acid:water (12:3:5) for up to 60 h.

Staining and elution methods. Electrophoretograms and chromatograms were stained with silver nitrate (Fry, 2000) to reveal monosaccharides, oligosaccharides, alditols, saccharinic acids and phenols, and with aniline hydrogen phthalate to reveal monosaccharides and reducing disaccharides. The paper strips of interest from both methods were eluted by a syringe method with distilled H_2O (Eshdat & Mireman, 1972). The eluted material was incorporated into 1.5% (w/v) bacteriological agar with 0.2% (w/v) yeast extract.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD). Twenty microlitres each of the aqueous phase of the onion extract and the TFA-hydrolysed samples (0.1 mg ml^{-1}) were analysed by HPAE–PAD (Dionex). The system consisted of an AS3500 autosampler, GP40 gradient pump, ED40 electrochemical detector, and PC10 pneumatic controller. The amperometry detector cell contained a gold electrode and a pH-Ag|AgCl combination reference electrode. CarboPac MA-1, PA-1, and PA-100 columns and guard columns were used for the separation of alditols, monosaccharides, and oligosaccharides respectively. Eluents, degassed by bubbling with helium, were as follows. MA-1: 600 mM NaOH at 0.4 ml min^{-1} (isocratic); PA-1: 20 mM NaOH for 3 min, then H_2O for 32 min, then a 0→200 mM NaOH gradient over 10 min (all at 1.0 ml min^{-1} with post-column addition of base); PA-100: 100 mM NaOH throughout, supplemented with a 0→200 mM NaOAc gradient over 30 min, then 200→800 mM NaOAc over 10 min (all at 1.0 ml min^{-1}). Analytes were identified by comparison of retention times to those of standards and quantified by integration of peak area with Chromeleon software (Dionex).

Investigation of conserved EPS gene clusters in Bcc species. Genome sequences representing five Bcc species were examined to determine if two previously published EPS gene clusters within *B. cenocepacia* J2315, the *bce* gene cluster (Moreira *et al.*, 2003) and the *wcb* gene cluster (Parsons *et al.*, 2003), are conserved across the Bcc. The amino acid sequences for every open-reading frame (ORF) within each of the two gene clusters within *B. cenocepacia* J2315 (genome sequence available at the Wellcome Trust Sanger Institute; <http://www.sanger.ac.uk/>) were used to search by TBLASTN the following Bcc genome sequences: *B. ambifaria* AMMD, *B. vietnamiensis* G4, *B. multivorans* ATCC17616, *Burkholderia* sp. 383. (all sequences are available at the US Department of Energy Joint Genome Institute; <http://www.jgi.doe.gov/>) and *B. dolosa* AU0158 (available at The Broad Institute; <http://www.broad.mit.edu/>). TBLASTN searches were performed using default parameters (BLOSUM 62, Word size 3).

RT-PCR analysis of EPS biosynthetic gene clusters. The EPS-producing strain, *B. ambifaria* AMMD, was cultured in 0.2 % (w/v) yeast extract with or without supplementation with 2 % (w/v) mannitol. RNA was extracted from mid-log phase cultures (RNeasy Protect Bacteria Mini Kit; Qiagen) and DNase I-treated (RNase-free DNase set; Qiagen), prior to reverse transcription with 1.5 µg RNA template, random primers and SuperScript III Reverse Transcriptase (Invitrogen). cDNAs and corresponding non-RT controls were used as template in PCR reactions specific for two distinct *wza* homologues (Bamb_5549 and Bamb_3621) located within two separate putative EPS biosynthetic gene clusters. Primer sequences are available upon request.

PCR analysis of *bceB* gene. Using the *bceB* gene sequence of *B. cenocepacia* J2315, *bceB* homologues were identified within the publicly-available genome sequences of *B. ambifaria* AMMD, *B. cepacia* sp. 383, *B. cenocepacia* AU1054, *B. cenocepacia* HI2424, *B. cenocepacia* PC184, *B. vietnamiensis* G4 and *B. dolosa* AU0158. The eight gene sequences were aligned, and PCR primers flanking the location of the previously described 11-bp deletion in *B. cenocepacia* J2315 (Moreira *et al.*, 2003) were designed based on conserved regions across the eight aligned sequences (For 5'-TGAAGGCGGT[G/C]GCGATCGTC; Rev 5'-TCGAT[G/C]CGCACGTCGTCGAG). For preparation of genomic DNA, 1-2 bacterial colonies were resuspended in 20 µl lysis solution [0.25% (w/v) SDS, 0.05 M NaOH] and incubated at 95 °C for 15 min. After brief centrifugation, 180 µl sterile water was added and centrifugation performed at 13,000 g for 5 min. Two microlitres of supernatant containing genomic DNA was used as template in PCR assays. PCR reactions were performed in a 50 µl volume containing 300 nM forward and reverse primer, 1.5 mM MgCl₂, 260 µM of each dNTP, 4% (v/v) DMSO, 1 U Taq polymerase (Invitrogen) and appropriate manufacturer's reaction buffer. Thermal cycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems) with the following parameters: 94 °C 3 min; 40 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s); 72 °C 10 min. PCR products were electrophoresed on a 4 % E-Gel (Invitrogen) and visualised by UV illumination. The *B. cenocepacia* J2315 *bceB* gene sequence harbouring the 11-bp deletion yields a PCR product of 140 bp, compared to 151 bp from the wildtype sequence found in *B. cenocepacia* IST 432 (Videira *et al.*, 2005).

PCR analysis of BCESM and *cblA* gene. Genomic DNA was prepared as described above. PCRs were performed as described previously (Mahenthiralingam *et al.*, 1997; Sajjan *et al.*, 1995).

Insertional inactivation of *bceB* in *B. ambifaria* AMMD. Insertional inactivation of *bceB* was performed using the pGPΩTp suicide vector, essentially as described previously (Flannagan *et al.*, 2007). In brief, a 300-bp fragment internal to the *bceB* ORF of *B. ambifaria* AMMD and flanked by *Xba*I and *Eco*RI sites was PCR-amplified and ligated into the corresponding sites in pGPΩTp following appropriate restriction. Resulting plasmids were transformed into *E. coli* GT115 competent cells (InvivoGen, San Diego USA) and subsequently introduced into *B. ambifaria* AMMD by triparental mating. Resulting exconjugants were selected using gentamicin (50 mg l⁻¹) and trimethoprim (100 mg l⁻¹), and mutants identified by PCR using a chromosomal-specific primer in conjunction with the vector-specific primer RSF1300 (Flannagan *et al.*, 2007).

Results

Exopolysaccharide production on onion agar

When a lyophilised onion extract was incorporated into agar at 2% (w/v) as the sole nutrient, members of the Bcc were not only able to grow, but a majority of isolates also produced copious amounts of exopolysaccharide as typified by *B. ambifaria* AMMD (Fig. 1 (a) and (b)). This phenotype is not observed when the Bcc are cultured on *Burkholderia cepacia* media (Mast Diagnostics), nutrient agar and other common laboratory media. Induction of EPS biosynthesis on onion agar was observed in all Bcc species investigated, but was not observed in all strains within a species (Table 1 and Supplementary Tables 1, 2 and 3). The exception was the single strain of *B. stabilis* available for testing. Of particular interest was the failure of the well-characterised *B. cenocepacia* ET12 representatives J2315, K56-2 and BC7 to produce EPS on onion agar. In addition, there was no correlation between induction of Bcc EPS on onion agar and the ability of individual Bcc strains to cause maceration of onion bulbs (data not shown).

The onion factor

Attempts were made to identify the “onion factor” responsible for inducing EPS biosynthesis by use of standard biochemical methods for extracting and fractionating phytochemicals. The causative factor was retained on drying *in vacuo*, and remained in the aqueous phase after partition with ethyl acetate at pH 7.0 and pH 2.0. When further physiochemical analyses discounted the role of proteins and lipids, attention was turned to the carbohydrate content of onion extract. After preparative paper electrophoresis in buffers at pH 2.0 and 6.5, the only biological activity recovered from paper strips co-migrated with the standard glucose, indicating the absence of ionisable functional groups such as phosphate, acid or amine (data not shown). Analytical paper chromatography and HPAE-PAD identified the major carbohydrate components as sucrose, glucose, fructose and fructans (Fig. 2). The HPAE-PAD chromatograms in Fig. 2 show characteristic peaks of glucose, fructose and sucrose. Sucrose breaks down to fructose and glucose upon mild hydrolysis, as do the fructans to fructose. Fructose breaks down under complete hydrolysis as expected for a ketose sugar, whilst the aldose sugar glucose remains stable. The ability of these and related compounds to stimulate EPS biosynthesis in Bcc was then investigated (Table 1). Glycerol and mannitol were included as these sugar alcohols have previously been noted to enhance EPS biosynthesis in *Pseudomonas aeruginosa* (Whitchurch *et al.*, 1996) and the Bcc (Sage *et al.*, 1990; Zlosnik *et al.*, 2008). Glucitol was included because of its close degradative relationship with fructose and mannitol (Allenza *et al.*, 1982). These experiments showed that within a particular Bcc species, EPS biosynthesis was strain-specific and that the most potent inducers of EPS were fructose and all alditols tested, most significantly mannitol and glucitol, as well as the cyclitol *myo*-inositol. Importantly, the profile of EPS biosynthesis production with these sugars was similar to that observed with onion extract

(Table 1). EPS biosynthesis was not observed on agar containing yeast extract alone, nor in the presence of glucose (Table 1), galactose, lactose or maltose (data not shown) with any Bcc strains tested.

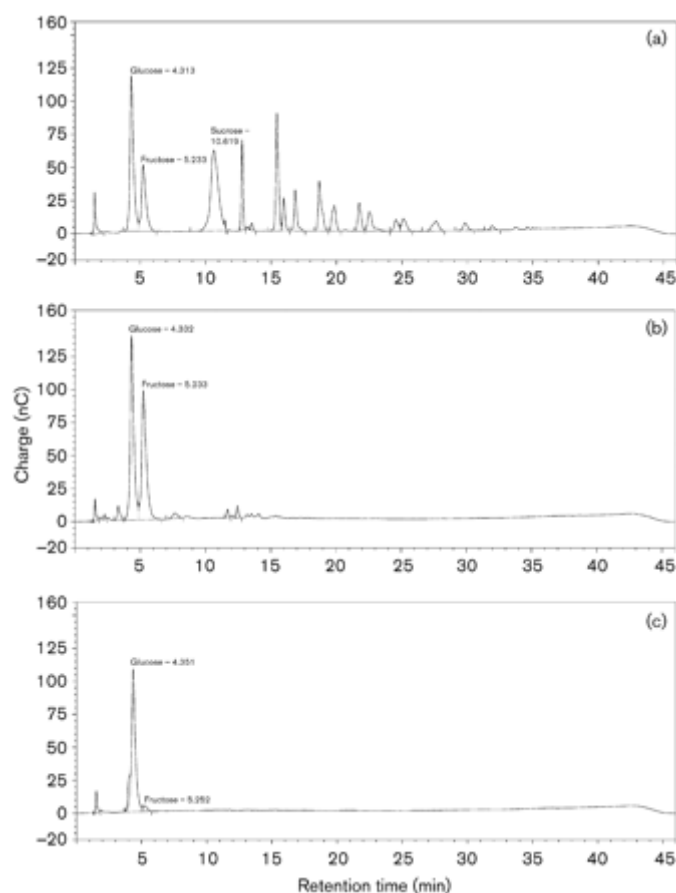


Figure 2. HPAE-PAD chromatograms: Carbowpac PA-100 column separation of sugars in onion extract. (a) Crude onion extract; (b) onion extract hydrolysed by 2 M TFA 60 °C 1 h; (c) onion extract hydrolysed by 2 M TFA 120 °C 1 h. Glucose, fructose and sucrose peaks were clearly identified based on standards (not shown), and hydrolysis pattern. Peaks with retention times between 12 and 36 min appear to be oligosaccharides of fructose based on their degradation to fructose under mild hydrolysis.

Investigation of the molecular basis for EPS biosynthesis

With the exception of the single *B. stabilis* strain tested, all Bcc species were shown to be capable of producing EPS when grown on onion agar (Table 1 and Supplementary Tables 1-3), suggesting the presence of a conserved EPS biosynthetic gene cluster. Consequently, genome sequences representing five Bcc species (*B. ambifaria*, *B. multivorans*, *B. vietnamiensis*, *B. dolosa* and *Burkholderia* sp. 383) were examined to determine if two putative EPS gene clusters within *B. cenocepacia* J2315, the *bce* gene cluster (Moreira *et al.*, 2003) and the *wcb* gene cluster (Parsons *et al.*, 2003), are conserved across the Bcc. The *wcb* gene cluster was found to be poorly conserved, with between one-third and one-half of J2315 ORFs having no direct

homologues within the species examined (data not shown). Notably, within the EPS-producing *B. ambifaria* AMMD strain, over half of the J2315 *wcb*-associated ORFs have no homologues, and the remaining homologous ORFs are not organised within a gene cluster. In contrast, the *bce* gene cluster was conserved across all species examined, both in terms of sequence homology and organization of ORFs (Supplementary Figure 1). In the course of these genome comparisons, a third putative polysaccharide biosynthetic gene cluster was observed on chromosome 2 of *B. cenocepacia* J2315. This cluster encodes two putative EPS transporter proteins (BCAM1330 and BCAM1331), an acyltransferase (BCAM1333), several glycosyltransferases (BCAM1335, BCAM1337, BCAM1338), a polysaccharide biosynthesis protein (BCAM1336) and a mannose-6-phosphate isomerase (BCAM1340). This cluster is conserved amongst several *Bcc* species, albeit to a lesser extent than the *bce* gene cluster (data not shown). In the EPS-producing *B. ambifaria* AMMD, this gene cluster maps to ORFs Bamb_3621 through to Bamb_3629.

To investigate which polysaccharide gene cluster is induced by growth on mannitol, the expression of representative genes from two distinct EPS gene clusters was assessed in *B. ambifaria* AMMD grown in the presence and absence of mannitol. The genes studied each encode homologues of the Wza EPS export protein: Bamb_5549 of the *bce* gene cluster (equivalent to *bceE*, BCAM0858 of *B. cenocepacia* J2315), and Bamb_3621 of the novel putative polysaccharide gene cluster described above (BCAM1330 of *B. cenocepacia* J2315). As shown in Figure 3, expression of Bamb_3621 was not observed under either growth condition. In contrast, expression of the *bceE* homologue (Bamb_5549) was clearly induced by the presence of mannitol. Consequently, we focused on the *bce* gene cluster to investigate why some *Bcc* isolates, most notably those of the *B. cenocepacia* ET12 lineage, failed to produce EPS under any growth conditions.

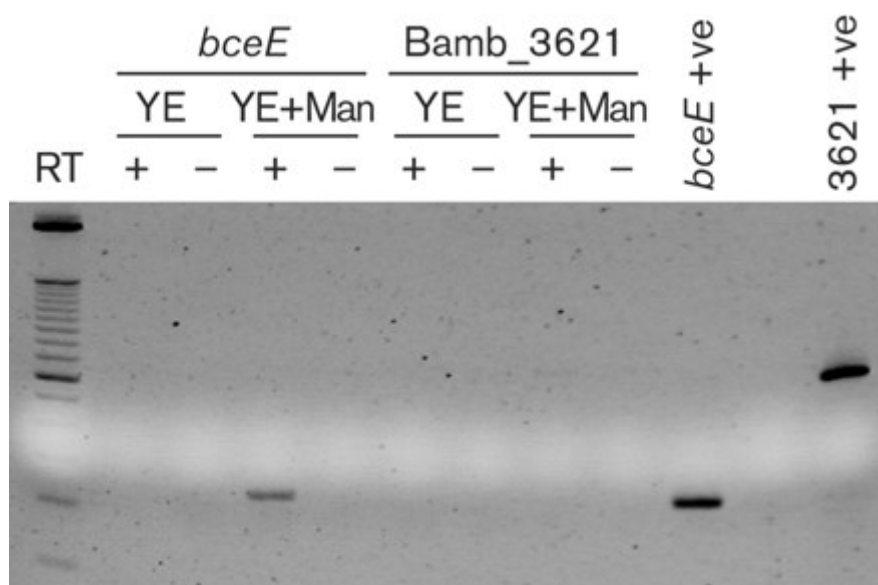


Figure 3. Induction of the *bce* gene cluster by mannitol. RT-PCR analysis was performed to assess the expression of two distinct *wza* homologues (*bceE* and Bamb_3621) of *B. ambifaria* AMMD in the absence and presence of mannitol. The *bceE* gene (Bamb_5549) is located within the *bce* gene cluster, whilst Bamb_3621

is located within a distinct putative EPS biosynthetic gene cluster. Expression was assessed in yeast extract (YE) and yeast extract supplemented with 2% (w/v) mannitol (YE+Man). Expression of Bamb_3621 was not detected in YE or YE+Man. In contrast, *bceE* expression was strongly induced by growth in YE+Man. For each sample, RT and non-RT reactions (+/-) are shown alongside each other. Genomic DNA positive controls are shown for each gene (*bceE* +ve, *3621*+ve).

A PCR assay was designed to screen isolates for an 11-bp deletion in the *bceB* gene which has been suggested to be responsible for loss of EPS production in the CF isolate *B. cenocepacia* J2315 (Moreira *et al.*, 2003). Of the panel of strains shown in Table 1, only the *B. cenocepacia* ET12 isolates J2315, K56-2 and BC7 harbour the 11-bp deletion (data not shown). This result prompted us to test a panel of *B. cenocepacia* IIIA strains containing both ET12 and non-ET12 isolates. There was a clear correlation between the 11-bp deletion and the presence of both *cblA* (cable pilus) and BCESM (*B. cepacia* epidemic strain marker), indicating this deletion is a conserved feature within the ET12 lineage (Fig. 4). In our study, with the exception of strain E3051, the presence of the deletion correlated with the lack of EPS production in all *B. cenocepacia* IIIA isolates examined (Fig. 4; Supplementary Table 1). Furthermore, the 11-bp deletion in the *bceB* gene was not observed in any *B. cenocepacia* IIIB or *B. multivorans* isolates studied (see Supplementary Tables 2 and 3).

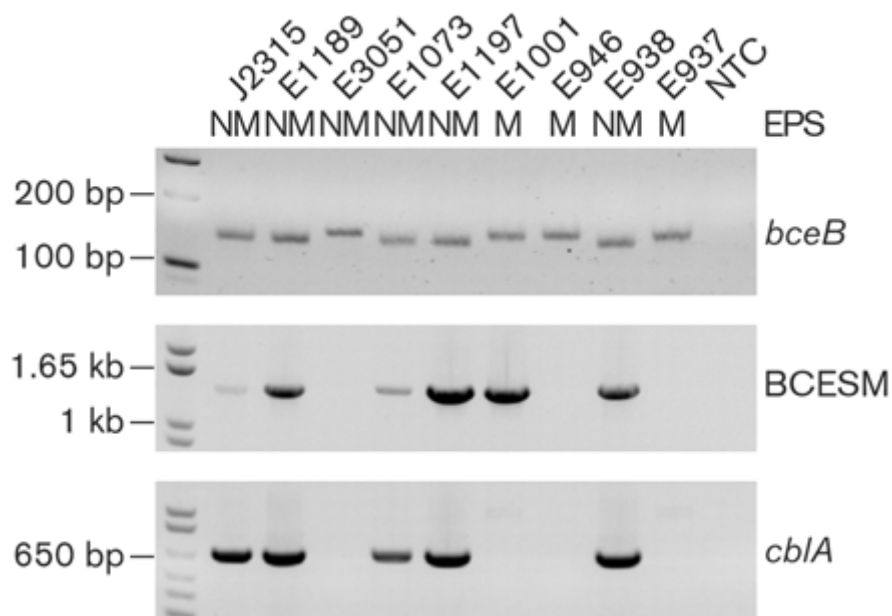


Figure 4. *bceB* PCR analysis of representative *B. cenocepacia* IIIA isolates. For each isolate, EPS production is indicated as mucoid (M) or non-mucoid (NM) alongside PCR analysis of *bceB*, and the epidemic strain markers *cblA* and BCESM. All PCR products are pictured alongside the 1 kb Plus DNA Ladder (Invitrogen). NTC, no template control. The *bceB* PCR assay yields a 151 bp product from wild-type sequence and a 140

bp product from sequence harbouring an 11 bp deletion (as previously reported within J2315). With one exception (E3051), lack of EPS production within *B. cenocepacia* IIIA isolates correlated with the 11 bp deletion within *bceB*. This deletion was only observed within strains that were positive for both *cblA* and BCESM, indicating it to be a conserved feature of the ET12 lineage. The *bceB* deletion was not observed in a strain harbouring just one of the two epidemic strain markers (E1001).

Using proven methods for the complementation of gene function in *B. cenocepacia* (Ortega *et al.*, 2005), attempts were made to complement BceB function within *B. cenocepacia* K56-2 by introducing a wildtype *bceB* ORF amplified from *B. cenocepacia* PC184. Whilst expression of 6His-tagged BceB was detected in *E. coli* C41(DE3) cells, previously shown to support expression of membrane-bound proteins (Miroux & Walker, 1996), we were unable to detect expression within *B. cenocepacia* K56-2 (data not shown). Consequently, we chose to disrupt the *bceB* ORF of an EPS-producer. Insertional inactivation of the *bceB* gene resulted in loss of EPS production in *B. ambifaria* AMMD when grown mannitol agar (Fig.1 (c)) and on onion agar (data not shown).

Discussion

In the present study, we returned to the original Bcc host and report that Bcc isolates previously considered nonmucoid produce copious amounts of EPS when onion tissue is provided as the sole nutrient, highlighting the metabolic potential of this group of organisms. This novel and surprising observation is not species-specific, and is exhibited by isolates of both clinical and environmental origin. The onion components responsible for EPS induction are primarily the carbohydrates sucrose, fructose and fructans. Additionally, all alcohol sugars tested are able to induce EPS production, in particular mannitol and glucitol.

The reasons why EPS biosynthesis is readily induced by onion extracts and, in particular, by fructose and the hexitol sugars mannitol and glucitol require further investigation. An ability to respond to inulin was confined to certain strains, all of which responded well to fructose. This suggests that the fructan polysaccharide was only active in bacteria that can hydrolyse it to fructose. The ability of one strain (BTS7) to respond particularly strongly to inulin could possibly be due to its enhanced ability to hydrolyse inulin to fructose. Interestingly, the similar profile of EPS induction shown by fructose and alcohol sugars agrees well with a previous study which showed that the initial steps of utilisation of these sugars in the Bcc differ from those in most other pseudomonads. Growth of Bcc on fructose involves active transport followed by fructokinase conversion to fructose 6-phosphate then degradation via the Entner–Doudoroff (Allenza *et al.*, 1982). Growth on mannitol and glucitol also requires this pathway following active uptake and intracellular oxidation to fructose.

The role of Bcc EPS as a putative virulence factor is unclear. The influence of mannitol and other alcohol sugars on EPS production suggests that the prevalence of EPS in the Bcc may have been underestimated. In addition, given the variety of human, animal, plant and other models studied, the role of EPS may be influenced by the host involved and the route of administration. EPS has been associated with altered Bcc clearance in a mouse model of infection (Conway *et al.*, 2004), and EPS-deficient Bcc mutants displayed reduced mortality within a CGD mouse model (Sousa *et al.*, 2007). Similarly, a role for EPS in persistence in human airways is suggested by its capacity to scavenge reactive oxygen species and inhibit neutrophil chemotaxis (Bylund *et al.*, 2006). In our study, EPS biosynthesis did not correlate with the ability to cause onion rot, which is perhaps to be expected since pectinases rather than EPS are likely to play the major role in the maceration of plant tissue. Cunha and colleagues (2004) did not observe a clear correlation between EPS biosynthesis *in vitro* and the ability of Bcc strains to establish chronic infections within the CF lung. Recent evidence, however, suggests a subtle and unexpected role for Bcc EPS in CF lung infection. Consistent with our findings, Zlosnik and colleagues (2008) reported that isolates of *B. cenocepacia*, the most virulent Bcc species, are most frequently nonmucoid. They also observed a mucoid to nonmucoid conversion in sequential isolates of Bcc from chronically infected CF patients. This apparent loss of mucoidy *in vivo*, and its absence in the virulent *B. cenocepacia* ET12 lineage, provides an intriguing contrast with the characteristic nonmucoid to mucoid conversion observed with alginate-producing *P. aeruginosa*. Zlosnik and colleagues suggest that Bcc EPS could be responsible for the persistence of Bcc in CF airways whilst loss of EPS leads to increased disease severity.

In our investigation of EPS biosynthesis determinants, we focussed on the highly conserved *bce* gene cluster. Previously described by Moreira and colleagues (2003), the *bce* gene cluster has had several of its encoded proteins characterized (Ferreira *et al.*, 2007; Sousa, 2007; Videira *et al.*, 2005) and is thought to be involved in cepacian biosynthesis. In the present study, we demonstrated induction of the *bce* gene cluster by mannitol, and found a clear correlation between the inability of representatives of the *B. cenocepacia* ET12 lineage to produce EPS and the presence of an 11-bp deletion within the *bceB* gene, originally described within the genome sequence of *B. cenocepacia* J2315 (Moreira *et al.*, 2003). Consistent with this correlation, insertional inactivation of *bceB*, which encodes a glycosyltransferase, resulted in the loss of EPS production by *B. ambifaria* AMMD when grown on onion media. Combined, these observations highlight the pivotal role of the *bce* gene cluster in onion-induced EPS biosynthesis, and suggest that the observed EPS is cepacian. However, mutations elsewhere within the *bce* gene cluster, or in other EPS-related gene clusters, must be responsible for the lack of EPS biosynthesis in *B. cenocepacia* strain E3051 (Supplementary Table 1), and in other EPS-negative Bcc strains in our study which lack the 11-bp deletion in *bceB* (Supplementary Tables 2 & 3).

The ability of hexoses and hexitols, in particular mannitol, to enhance EPS biosynthesis in the *B. cepacia* complex has disturbing implications for therapeutic intervention in CF. Recent attempts to improve airway

clearance with hypertonic saline 5% (w/v) have been handicapped by the problem of salty taste and the salt-sensitive nature of many antimicrobial peptides. Thus, attention has turned to the use of non-ionic osmolytes, including inhaled mannitol (Daviskas *et al.*, 2008; Robinson *et al.*, 1999; Wills, 2007), which is marketed as BronchitolTM (Pharmaxis). Robinson and colleagues acknowledged that the majority of *P. aeruginosa* and Bcc isolates are able to utilise mannitol as a carbon and energy source. However, they felt that the nutritional influence of mannitol as a therapeutic osmolyte would be minimal given the abundance of other nutrients already present in CF respiratory secretions. On a cautionary note, they state that this potential problem would need to be confirmed by quantitative microbiology. The potential induction of virulence determinants during osmolyte therapy has to our knowledge not been considered. Our results also provide justification for the continued exclusion of CF individuals known to be infected with Bcc from ongoing trials of inhaled mannitol (ClinicalTrials.gov identifier NCT00117208 and NCT00251056).

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